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Role of IGF-I and Efn–Eph Signaling in Skeletal Metabolism

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Abstract

Insulin-like growth factor (IGF)-I and ephrin ligand (efn)–receptor (Eph) signaling are both crucial for bone cell function and skeletal development and maintenance. IGF-I signaling is the major mediator of growth hormone-induced bone growth, but a host of different signals and factors regulate IGF-I signaling at the systemic and local levels. Disruption of the *Igf1* gene results in reduced peak bone mass in both experimental animal models and humans. Additionally, efn–Eph signaling is a complex system which, particularly through cell–cell interactions, contributes to the development and differentiation of many bone cell types. Recent evidence has demonstrated several ways in which the IGF-I and efn–Eph signaling pathways interact with and depend upon each other to regulate bone cell function. While much remains to be elucidated, the interaction between these two signaling pathways opens a vast array of new opportunities for investigation into the mechanisms of and potential therapies for skeletal conditions such as osteoporosis and fracture repair.

Keywords

IGF-I; Efn; Eph; Bone

1. Introduction

Osteoporosis is a debilitating disease affecting millions worldwide. In the United States alone, more than 10 million people have osteoporosis, and that number is predicted to rise, increasing the burden of suffering as well as healthcare costs on individuals as well as society at large. By the year 2025, the annual rate of osteoporosis-related fractures in the

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Declaration of Interest

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United States has been projected to be greater than 3 million, leading to an economic burden of more than \$25 billion per year (Burge *et al.* 2007). Osteoporosis, a disease characterized by low bone mass and compromised skeletal microarchitecture, occurs when elevated rates of bone resorption are not sufficiently compensated for by increased bone formation. Frequently, osteoporosis occurs in the context of accelerated bone resorption due to sex hormone deficiency and aging in postmenopausal women. While several current therapeutic strategies have achieved some success at slowing the rate of bone loss, devastating osteoporotic fractures still occur at an alarming rate, emphasizing both the importance of achieving a high peak bone mass during development and the necessity of investigating the mechanisms regulating bone growth and maintenance in order to develop new therapies to treat and prevent osteoporosis. Much work has gone into understanding of the origin and functions of cartilage-forming chondrocytes, bone matrix-producing osteoblasts and osteocytes, and bone-resorbing osteoclasts, and these studies have revealed that the network of signals regulating these cells and their functions is complex. Indeed, there are many growth factors which contribute to the regulation of skeletal development. Two growth factor signaling pathways which exert many effects on skeletal development are the IGF-I and efn-Eph pathways. The focus of this review is to explore the extent to which each of these two signaling pathways has been studied with respect to its contribution to skeletal development and to review what is currently known about the ways in which the interactions of these two pathways may enhance our understanding of skeletal regulation and lead to opportunities to discover novel therapies for osteoporosis and bone repair.

2. IGF Signaling in Bone

Insulin-like growth factors (IGFs) are the most abundant growth factors in bone matrix and are critical regulators of bone growth and maintenance. IGF-I and -II are small peptide hormones which are structurally similar to insulin, and they act by binding the type I IGF receptor (IGF-IR), a receptor tyrosine kinase which is also structurally similar to the insulin receptor (Centrella *et al.* 1990; Slootweg *et al.* 1990). During intrauterine development, IGF-II is responsible for growth, while IGF-I predominantly regulates skeletal growth and maintenance during postnatal life. As a member of the growth hormone (GH)/IGF axis, IGF-I is responsible for mediating a significant proportion of GH's effects on bone (Fig. 1).

a. GH regulation of IGF-I

The systemic, endocrine actions of IGF-I are largely due to GH-induced hepatic IGF-I secretion into the circulation. In fact, conditional hepatic deletion of the *Igf1* gene (Sjögren *et al.* 1999; Yakar *et al.* 1999) or GH receptor (*Ghr*) gene (Fan *et al.* 2009) in mice resulted in up to 90% reductions in systemic circulating IGF-I levels. Furthermore, up to 75% of circulating IGF-I is bound up in a ternary complex with IGF binding protein 3 (IGFBP3) and an acid-labile subunit (ALS) which is also produced in the liver in response to GH signaling. Growth hormone-induced hepatic secretion of circulating, endocrine-acting IGF-I may be responsible for up to 30% of body weight (Stratikopoulos *et al.* 2008). However, IGF-I also acts locally in an autocrine/paracrine manner. An additional 39% of body weight may be due to local IGF-I production, of which 4% may be accounted for by GH-induced IGF-I production in peripheral tissues (Stratikopoulos *et al.* 2008).

Since IGF-I is known to interact with efn–Eph signaling and IGF-I mediates many of GH's effects on bone, this review focuses primarily on the contribution of IGF signaling to skeletal development. However, the entire GH/IGF axis is crucial for skeletal development, and other recent reviews have examined the GH/IGF axis as a whole (Locatelli & Bianchi 2014; Lindsey & Mohan 2015; Liu *et al.* 2016). Furthermore, some evidence suggests that the GHR and downstream effectors of GH signaling Janus kinase 2 (JAK2) and signal transducer and activator of transcription 5B (STAT5B) may complex with EphA4 to enhance *Igf1* expression, suggesting a more complex relationship between the GH/IGF axis and efn–Eph signaling which deserves further investigation (Jing *et al.* 2012; see below).

b. Systemic regulation and effects of IGF-I

Many systemic signals in addition to GH are known to regulate IGF-I. For example, circulating parathyroid hormone (PTH) is known to enhance osteoblast proliferation, differentiation, and survival by increasing local production of IGF-I (Linkhart & Mohan 1989; McCarthy *et al.* 1989; Miyakoshi *et al.* 2001a; Bikle *et al.* 2002; Yamaguchi *et al.* 2005). Additionally, sex steroids cause an increase in IGF-I expression during puberty (Christoforidis *et al.* 2005; Veldhuis *et al.* 2005). In fact, crosstalk between the sex steroid and GH/IGF-I axes is crucial for linear bone growth and acquisition, and GH/IGF-I may contribute to the development of skeletal sexual dimorphism (Liu *et al.* 2016). Furthermore, thyroid hormone (TH) increases both hepatic and skeletal IGF-I expression during a critical prepubertal growth period in mice in which TH is a more critical regulator of skeletal growth than GH; thus, IGF-I is thought to mediate many TH effects on the skeleton (Xing *et al.* 2012; Cheng *et al.* 2016). Conversely, glucocorticoids and 1,25-dihydroxyvitamin D3 [1,25-(OH)₂D₃] have been shown to downregulate IGF-I expression (Chen *et al.* 1991; Scharla *et al.* 1991; Canalis 2005). Thus, there is ample evidence in the literature to suggest that many of the systemic effects of calcium regulating hormones on bone are mediated in part via regulation of IGF actions.

c. Local regulation and effects of IGF-I

At the local level, many growth factors and other signals in addition to GH are involved in regulating IGF expression. For example, fibroblast growth factor (FGF) 2, transforming growth factor (TGF)- β 1, bone morphogenetic protein (BMP) 7, and interleukin-1 are known to influence expression of IGF-I in bone cells (Tremollieres *et al.* 1991; Knutsen *et al.* 1995; McCarthy & Centrella 2001; Zhang *et al.* 2002a). These studies have shown that the actions of many of the local growth factors on IGF-I expression vary depending on both cell type and stage of differentiation.

IGF activity, however, is dependent upon more than just the regulation of IGF expression levels. IGF-I interacts with several IGF binding proteins (IGFBP-1 through -6) which serve to regulate IGF-I activity at both the systemic and local levels. Up to 75% of the IGF-I in plasma is bound up in a ternary complex with IGFBP-3 and an acid-labile subunit (ALS), and in this way IGFBP-3 regulates the concentration of active, free IGF-I available in circulation for signaling (Bagi *et al.* 1994; Rosen *et al.* 1994; Jones & Clemmons 1995; Rajaram *et al.* 1997). Not all IGFBPs affect IGF-I signaling in the same way; generally, IGFBP-3 and -5 enhance IGF-I's effect on osteoblasts by prolonging its half-life in

circulation while IGFBP-1, -2, -4, and -6 inhibit IGF-I's effect on osteoblasts by preventing its interaction with the type I IGF receptor (IGF-IR) (Govoni *et al.* 2005). Moreover, IGFbps themselves are regulated at the expression level by systemic and local factors including PTH, 1,25-(OH)₂D₃, glucocorticoids, estrogen, retinoic acid, IGFs, TGF- β , BMPs, PDGF, and interleukins (Knutsen *et al.* 1995; Chevalley *et al.* 1996; Gabbitas & Canalis 1996; Honda *et al.* 1996; Hayden *et al.* 1997; Malpe *et al.* 1997; Kveiborg *et al.* 2001; Denger *et al.* 2008; DeMambro *et al.* 2015). IGFBP activity is also regulated by IGFBP proteases including pregnancy-associated plasma protein (PAPP)-A, PAPP-A2 (Kanzaki *et al.* 1994; Conover 1995; Qin *et al.* 2006; Tanner *et al.* 2008; Christians *et al.* 2013), and ADAM-9 (Mohan *et al.* 2002). Regulation of IGF activity by IGFbps and, by extension, regulation of the IGFbps themselves are therefore further crucial determinants of IGF signaling in bone.

d. IGF-I and Mechanotransduction

Considerable experimental evidence indicates that mechanical strain is a key physiological regulator of bone formation. IGF-I plays an important role in mechanotransduction and the skeletal response to mechanical strain (Tian *et al.* 2017). Mechanical loading leads to a rapid increase in IGF-I expression in bones (Lean *et al.* 1995; Xing *et al.* 2005; Reijnders *et al.* 2007), an effect which is mediated via an integrin-dependent phosphorylation of the IGF-I receptor (IGF-IR) (Kapur *et al.* 2005; Lau *et al.* 2006). *In vivo* overexpression of *Igf1* in mouse osteoblasts led to increased periosteal bone formation in response to low-magnitude loading (Gross *et al.* 2002), and osteoblasts exposed to dynamic strain demonstrate increased activation of PI3K/Akt and β -catenin, downstream effectors of IGF-I signaling (Sunters *et al.* 2010). Furthermore, conditional knockout of *Igf1* in osteoblasts expressing type I collagen (Col-I, Kesavan *et al.* 2011) or osteocytes (Lau *et al.* 2013) prevented an osteogenic response to mechanical loading. In osteocytes, lack of *Igf1* disrupted mechanical strain-induced changes in expression of sclerostin and Wnt10b (Lau *et al.* 2013). In addition to its contributions to the response of osteoblasts and osteocytes to mechanical strain, IGF-I signaling may even play a role in mediating the ability of mechanical forces to regulate the proliferation, survival, and osteogenic differentiation of bone marrow derived mesenchymal stem cells (Sakata *et al.* 2003, 2004; Chen *et al.* 2017; Wang *et al.* 2017). Thus, IGF-I signaling is essential for the skeletal response to mechanical stimuli.

e. Nutrition and IGF-I

Diet and nutritional status are additional important regulators of IGF-I (Bonjour 2016). Under conditions of restricted dietary intake, IGF-I levels are reduced via several mechanisms: hepatic GH receptors are reduced in number, decreasing IGF-I production, and rates of IGF-I degradation and clearance are increased (Thissen *et al.* 2004). Furthermore, osteoblasts appear to be resistant to IGF-I action in the context of protein restriction (Bourrin *et al.* 2000). This IGF-I response to alternations in dietary protein intake specifically has been confirmed in both *in vivo* animal studies (Ammann *et al.* 2000; Bourrin *et al.* 2000) and patients after hip fracture (Schürch *et al.* 1998). The interaction of dietary protein and IGF-I may thus influence skeletal health on a number of levels including attainment of peak bone mass (Bonjour *et al.* 2007), skeletal response to physical activity (Chevalley *et al.* 2008), and bone loss in the context of calorie-poor conditions such as intensive exercise and anorexia nervosa (Russell *et al.* 1994; Grinspoon *et al.* 2000; Gremion

et al. 2001; Warren *et al.* 2002; Misra *et al.* 2008). Additionally, epidemiological studies indicate that higher protein intake may be protective against bone loss, musculoskeletal deterioration, and decreased IGF-I levels, particularly in the elderly (Dawson-Hughes *et al.* 2004; Gaffney-Stomberg *et al.* 2009; Bonjour 2016). Adequate nutrition and protein intake is thus crucial for skeletal development and maintenance.

f. Transgenic mouse models of IGF-I action

The question of the relative contributions of endocrine versus local IGF-I action to skeletal growth and maintenance has also been extensively studied. Several transgenic mouse models indicate that systemic IGF-I has a much larger effect on cortical bone size and expansion than on longitudinal bone growth. Mice with conditional disruption of hepatic *Igf1* (Sjögren *et al.* 1999; Yakar *et al.* 1999) or GH receptor (*Ghr*; Fan *et al.* 2009) had little to no reduction in linear bone growth despite up to 90% reductions in circulating IGF-I levels. Furthermore, mice with conditional disruption of hepatic IGF-I along with total ALS or total ALS and total IGFBP-3 had up to 97.5% decreases in circulating IGF-I levels; these mice had relatively small changes in body length, but the triple negative mice had a 50% reduction in cortical bone (Sjögren *et al.* 1999; Yakar *et al.* 1999, 2002, 2009), a similar reduction to that seen in mice lacking total IGF-I (Mohan *et al.* 2003). However, restoration of hepatic IGF-I production in total *Igf1* knockout mice rescued body size by approximately 30% (Stratikopoulos *et al.* 2008), indicating that endocrine IGF-I action may play a role in skeletal growth (Nordstrom *et al.* 2011; List *et al.* 2014).

Local expression of IGF-I is also critical for bone function. Overexpression of IGF-I in mouse osteoblasts *in vivo* has led to increased osteoblast activity, bone formation, and bone remodeling (Zhao *et al.* 2000; Jiang *et al.* 2006), and overexpression of regulators of IGF bioavailability including IGFBPs and their proteases suggests that local availability of IGF-I is an important determinant of bone formation (Miyakoshi *et al.* 1999; Richman *et al.* 1999; Zhao *et al.* 2000; Miyakoshi *et al.* 2001b; Devlin *et al.* 2002; Qin *et al.* 2006). Conditional disruption of *Igf1* and *Igf1r* specifically in osteoblasts confirmed that, while serum levels of IGF-I were unchanged, a lack of local IGF-I significantly impaired bone mineral density (BMD), bone size, and bone formation measures (Zhang *et al.* 2002b; Govoni *et al.* 2007a). Furthermore, local IGF-I expression in chondrocytes is also necessary for skeletal development; chondrocyte-specific *Igf1* disruption in mice decreased bone length and BMD and impaired growth plate organization and function (Govoni *et al.* 2007b; Wang *et al.* 2011, 2015). Thus, both systemic and local IGF-I contribute significantly to skeletal growth and maintenance.

g. IGF-I and osteoporosis

It has been well established that growth hormone (GH) secretion decreases with age (Corpas *et al.* 1993; Müller *et al.* 1999). Accordingly, levels of IGF-I in the serum and bone decrease with age as well (Benbassat *et al.* 1997; Seck *et al.* 1999). However, in addition to decreased levels of IGF-I with age, osteoprogenitor cells from aged rats do not respond as effectively to IGF-I (Tanaka & Liang 1996), indicating that the skeleton is particularly susceptible to deterioration with age. In fact, age is a major risk factor for osteoporosis, and IGF-I may play an important mechanistic role in explaining age-related bone loss.

Epidemiological evidence suggests that IGF-I levels are well correlated with bone mineral density in men and postmenopausal women (Muñoz-Torres *et al.* 2001; Gillberg *et al.* 2002), and a cross-sectional study indicated that elderly women who had sustained femoral neck fractures had significantly decreased levels of IGF-I, IGF-II, IGFBP-3, and IGFBP-5 (Boonen *et al.* 1999). Moreover, several recent studies have also observed relationships between deficiencies in components of the IGF system and increased rates of fracture in both women and men (Ohlsson *et al.* 2011; Paccou *et al.* 2012; van Varsseveld *et al.* 2015; Lundin *et al.* 2016). Recombinant human IGF-I has even undergone clinical trials as an osteoporosis treatment, with mainly positive results (Locatelli & Bianchi 2014). However, additional studies have reported little to no effect of IGF-I levels on fracture risk (Kassem *et al.* 1994; Seck *et al.* 1999; Gillberg *et al.* 2001; Martini *et al.* 2001; Zofková *et al.* 2001). Thus, while IGF-I may play a role in age-related bone loss and osteoporosis, further studies are needed to elucidate its precise contribution. Taken together, both animal studies and human clinical studies dealing with different aspects of bone formation have established IGF-I as a critical mediator of bone growth and homeostasis.

3. Ephrin/Eph Signaling in Bone

The ephrins are large families of membrane-bound ligands (efn: ephrin family receptor interacting proteins) and receptor tyrosine kinase receptors (Eph: erythropoietin-producing human hepatocellular receptors) that mediate cell–cell communication within and between tissues in a wide variety of biological functions. Efn–Eph mediate cell proliferation and migration, and they are critical in the regulation of adhesion, repulsion, and tension which segregates cells to establish and maintain tissue boundaries during development (Cayuso *et al.* 2015). The efn and Ephs are broadly expressed during tissue development and repair. These functions have also implicated the efn and Ephs in the dysregulated adhesion and motility that promotes tumor metastasis.

There are two families of ephrin ligands. In mammals, they comprise the five-member ephrin A (efnA) family of glycosylphosphatidylinositol (GPI)-linked ligands and the three-member ephrin B (efnB) family of transmembrane ligands. Efn-binding receptors are all receptor tyrosine kinase (RTK) receptors, but they belong to either the nine-member A receptor (EphA) family or the five-member B receptor (EphB) family. Both EphA and EphB have an extracellular efn-binding domain attached to two fibronectin-III repeats and the intracellular tyrosine kinase, sterile alpha motif (SAM) and postsynaptic density protein 95/disks large/zonula occludens-1 (PDZ)-binding domains. Ligand–receptor interactions can be promiscuous within the ligand–receptor family, but, with few exceptions, signaling is restricted between ligands and receptors within either the A or B family.

a. Forward and reverse signaling

EfnB and EphB regulation of tissue development and homeostasis is noteworthy because this family can signal in the forward direction, from ligand through receptor, as well as in the reverse direction, from receptor through ligand, through a PDZ binding motif connected to the intracellular cytoplasmic domain. Reverse signaling from EphA through efnA is not

well characterized, but this pathway might utilize regulatory proteins recruited to efnA clusters (Davy *et al.* 1999).

Eph receptor and binding diversity permits a wide array of forward and reverse signaling options to mediate their functions. EphA regulates the JAK/STAT pathway, while EphB regulates PI3 kinase-mediated proliferation. Activation of the Rho GTPases by EphA or EphB mediates actin effects on cell shape and movement. Activation of the Ras GTPase pathways generally leads to negative regulation of the MAP kinase pathway, resulting in reduced proliferation and migration by EphA as well as reduced adhesion by EphB (Edwards & Mundy 2008). Reverse signaling from EphB2 through efnB1 promotes stromal cell differentiation to osteoblasts. In this case, PDZ domains from efnB1 and the PDZ-containing protein Na/H exchange regulatory factor 1 (NHERF1) form a complex with protein tyrosine phosphatase (PTPN13) and the PDZ domain-binding transcriptional coactivator (TAZ) to promote *Osx* transcription and osteoblast differentiation (Xing *et al.* 2010). Other studies have demonstrated the interaction of efn's with various adaptor proteins, further increasing the potential of efn–Eph signaling to interact with diverse signaling partners. For example, the SH2/SH3 adaptor Grb4 binds the cytoplasmic tail of efnB1. The SH3 domains of these adaptor proteins can in turn recruit a number of SH3-binding partners (e.g., Axin, Pak1, FAK, and Paxillin) to the efnB1 signaling complex and participate in propagating the reverse signal in a complex manner (Cowan & Henkemeyer 2001). The diversity of efn–Eph signaling functions is thus the product of the ability of efn–Eph to signal in both a forward and reverse manner and the presence of cross-talk between the several efn–Eph pathways, non-efn–Eph signaling pathways (e.g., the fibroblast growth factor pathway; Sawada *et al.* 2010), and different adaptor proteins. Thus, dissecting intracellular signaling pathway cross-talk among different cell types presents a challenge in characterizing efn–Eph effects.

Additionally, ligand and receptor functions can be diversified and adjusted by higher order ligand and receptor associations resulting from clustering. Clustering can involve the extracellular receptor domain as well as the intracellular RTK and SAM domains. Homooligomerization can follow interactions between Ephs that follow the formation of the initial ligand–receptor tetramer. Additionally, interfamily hetero-oligomers have been observed to cluster and produce different activation or inhibition states according to the relative levels of expression within the cluster (Janes *et al.* 2011; Jurek *et al.* 2016). Conformational changes following Eph binding, while limited when compared to other RTKs, might explain some of the interfamily binding between ligands and receptors (Janes *et al.* 2012). The role of proteolytic cleavage in further regulating efn–Eph signaling has been reviewed elsewhere (Atapattu *et al.* 2014). Additionally, splice variants in EphA7 have been observed; kinase domain deficient variants bias receptor function from repulsion toward adhesion (Holmberg *et al.* 2000). Considering the wide array of receptors, the ability to signal in forward and reverse directions, and the extracellular and intracellular variations, modulation of the efn–Eph system of cell signaling is indeed complex.

b. Mechanisms of Efn–Eph signaling in skeletal development

The efn and Ephs have been best characterized during the development of various murine tissues. Global knockout mouse studies have demonstrated that efnA2 and EphA7 mediate neural development by inhibiting proliferation. EfnB2 patterns the somites and regulates neural cell development (Davy & Soriano 2007). Neurogenesis, axonal guidance, and vasculogenesis are regulated by different efn and Ephs through both forward and reverse signaling (Zhang & Hughes 2006; Wilkinson 2014). Given their established functions in cell motility and adherence, it is not surprising that members of both the A and B families of ligands and receptors have been associated with neo-angiogenesis and invasiveness of several types of tumors (Brantley-Sieders & Chen 2004; Kumar *et al.* 2006), with deleterious effects attributed to their expression levels in the affected tissues (Campbell & Robbins 2008).

A variety of efn and Ephs are expressed in various tissues during the normal inflammatory response to injury and in chronic inflammation, where they mediate diverse processes such as vascular permeability and cell motility (Coulthard *et al.* 2012). EfnB1 and EphB2 expression is upregulated during skin wound repair and drives the re-epithelialization necessary for healing through the down-regulation of tight junctions, the release of adherens, and the reduction in actinomysin tension (Nunan *et al.* 2015). Efn and Ephs mediate growth factor-regulated angiogenesis during development, and they would also be expected to mediate the repair of skeletal tissues in a similar fashion.

The efn and Ephs are widely expressed in skeletal tissues, as they are in most tissues. The *efna2*, *Epha2*, *Epha4*, *efnb1*, and *efnb2* genes are expressed in osteoclasts induced to differentiate *in vitro* (Irie *et al.* 2009); several ligand and receptor genes of both the A and B families are expressed in differentiating osteoblasts *in vitro* (Zhao *et al.* 2006; Matsuo & Otaki 2012). *In vivo*, *efnb2* gene and efnB2 protein expression in osteoblasts and osteoclasts was promoted by PTH and parathyroid hormone-related peptide (PTHrP). The authors suggest that the anabolic effect of PTH and PTHrP could be mediated by efnB2 action on osteoblasts that express EphB (Allan *et al.* 2008). These characteristics of efn–Eph expression have important implications for the maintenance of homeostasis between osteoblasts and osteoclasts and the balance between bone formation and bone resorption.

Bone lineage motility and differentiation are regulated by efn–Eph binding. EfnB2 and EphB4 interactions mediate the mobilization from the bone marrow to the blood of efnB2-expressing bone marrow hematopoietic precursors through the EphB4-expressing sinusoidal cells. Antibody-dependent inhibition of this interaction and dominant-negative EphB4 receptor studies established that the mechanism was dependent on forward cell–cell signaling (Kwak *et al.* 2016). *In vitro* spreading and migration assays on human mesenchymal stem cells (MSCs) using soluble efnB and EphB established that forward signaling through EphB2 promoted MSC spreading, but reverse signaling through efnB inhibited MSC attachment and spreading. Moreover, differentiation of MSCs to the chondrogenic or osteogenic lineages was promoted by reverse signaling from EphB2 through efnB1 and efnB2 (Arthur *et al.* 2011). EfnB family regulation of bone marrow MSCs is important for several aspects of bone formation and possibly bone repair.

It was the observation that spontaneous efn and Eph mutations resulted in abnormalities in embryonic skeletal development that first implicated efn–Eph as important regulators in bone. Additional studies in mice with targeted deletions of different *efn* and *Eph* genes have further established the importance of efn–Eph signaling in skeletal development and characterized its effects in the regulation of bone cell differentiation as described below.

The developmental functions of efn–Eph in the skeleton have been best described in craniofacial patterning abnormalities. EphA4 has been demonstrated in *Twist1* and *Epha4* mutant mice to mediate *Twist1* regulation of osteogenic cell migration to the coronal sutures and to then exclude these cells from the sutures. A failure of EphA4 signaling produced craniosynostosis in these models (Ting *et al.* 2009). In mice with *efnb1* mutations, it is believed that X-linked mosaicism of efnB1 impaired cell sorting. Using signaling-deficient mutations, forward and reverse signaling between efnB1 and EphB, which mediate adhesion and repulsion in mesenchymal cell condensations, was demonstrated to regulate this aspect of craniofacial development (Davy *et al.* 2004). The calvarial defect phenotype in *efnb1* mutants was similar to that observed in neural crest cell sorting and was the result of impaired gap junction communication involving efnB1 in early osteogenic precursor development (Davy *et al.* 2006). Similar craniofacial abnormalities are produced by *efnb1* signaling mutations in human pathology (Compagni *et al.* 2003). However, other studies have implicated unidirectional forward signaling in this phenotype. EfnB1 patterning of palate development is accomplished through forward signaling interactions with EphB2 and EphB3 receptors, as demonstrated by combination *Ephb2* forward-signaling deficient mutants/*Ephb3* knockout mice, which developed the cleft palate. The defect resided in reduced proliferation (Risley *et al.* 2009) of the palatal mesenchymal cells mediated by these EphBs. Recent studies have postulated that the separation of the sutures results from efnB1 forward signaling mediated by Rho-associated protein kinase (ROCK). ROCK signaling modifies intracellular actin distributions and the resulting cortical tension of efnB1-expressing cells, maintaining the suture separating them from non-efnB1-expressing cells (O’Neill *et al.* 2016).

The efnA and EphA families also mediate communication between osteoblasts and osteoclasts. Interactions between efnA2 and EphA4 enhance osteoclast differentiation but inhibit osteoblast differentiation, as osteoblast differentiation was enhanced in *Epha4*-deficient osteoblasts in response to *efna2* overexpression (Irie *et al.* 2009). This effect must bias the initiation of remodeling away from bone formation, although multiple efn–Eph interactions may mediate this communication (Fig. 2). EfnA2 also interacts with EphA2 in promoting osteoclastogenesis to promote osteoporotic resorption; ovariectomy in rats increased efnA2–EphA2 signaling, osteoclast development, and trabecular bone deterioration, effects that were reduced *in vitro* by the inhibition of efnA2 and EphA2 expression and *in vivo* by estradiol treatment (Liu *et al.* 2017). These observations suggest efnA–EphA modulation as an alternative to hormone related therapy for osteoporosis.

Studies in mice with targeted disruption of the *Epha4* gene in osteoclasts have shown that EphA4 functions are diverse. EphA4 expression inhibited the activity but not the development of osteoclasts through the altered phosphorylation of stimulatory and inhibitory mediators of osteoclast activity (Stiffel *et al.* 2014). EphA5 has been determined to be an

inhibitor of osteogenic development of bone marrow stromal cells in culture (Yamada *et al.* 2013). EphA5 inhibition is overcome by the exogenous addition of dexamethasone (Yamada *et al.* 2016), suggesting potential interaction between a systemic hormone and a local growth factor in regulating osteogenesis, although the mechanism remains to be elucidated. Developmental studies in *Hoxa13* knockout mice implicate EphA7 expression in mediating *Hoxa13*-dependent mesenchymal cell adherence and chondrocyte condensations in the knockout autopods, which display digit fusions (Stadler *et al.* 2001). The EphAs therefore participate in different aspects of skeletal development and homeostasis.

Studies in *efnb1* knockout mice established that efnB1 can promote different aspects of bone homeostasis. An osteoblast (*Osx*)-specific knockout of *efnb1* resulted in altered long bone development, with impaired trabecular and cortical bone formation. A decrease in osteoblasts and increase in osteoclast formation suggests that efnB1 can mediate bone formation and resorption (Nguyen *et al.* 2016). Other investigations have determined the mechanism of intracellular signaling that mediate this effect. Transgenic overexpression of *efnb1* in murine osteoblasts increased osteoblast differentiation and mineralization *in vitro*, increased *Osx* expression and trabecular bone formation *in vivo*, and reduced osteoclastogenesis through EphB2 in response to mechanical loading of the long bones (Cheng *et al.* 2013). Further studies also examined the role of efnB1 in the osteoclast lineage and established that deletion of *efnb1* from myeloid cells increased osteoclastogenesis. Trabecular bone was reduced in the knockout mice without changes in bone formation. *In vitro* studies implicated reverse signaling of efnB1 through EphB2 as a mechanism for this effect and a role for efnB1 as a negative regulator of osteoclast differentiation (Cheng *et al.* 2012).

Deletion of *efnb2* in osteoblasts resulted in a significant reduction in bone formation in knockout mice *in vivo* and reduced mineralization and expression of late stage osteoblast differentiation markers *in vitro*. *Efnb2* deletion has been observed to increase apoptosis in the osteoblast lineage, suggesting yet another mode for this efn in promoting bone formation (Tonna *et al.* 2014).

c. Role of Efn–Eph in cell–cell communication

Multiple efn–Eph interactions mediate several aspects of bone development and homeostasis, but the efnB2–EphB4 ligand–receptor signaling pair is especially well-studied in the mediation of osteoblast–osteoclast communication that maintains a homeostasis between bone formation and bone remodeling in the skeleton (Edwards & Mundy 2008). *In vitro* and *in vivo* studies established that the efnB2–EphB4 ligand–receptor pair is especially important for bone homeostasis, as forward signaling from efnB2 through EphB4 promoted bone formation from osteoblasts, while reverse signaling from EphB4 through efnB2 inhibited osteoclastogenesis by suppressing NFATc1 (Zhao *et al.* 2006). In this case, an *in vivo* reduction in osteoblast development and bone formation would be expected in osteoblast marker-specific *Ephb4* knockout mice, while an increase in osteoclast development and bone resorption functions would be expected in osteoclast marker-specific *efnb2* knockout mice. However, the osteoclast-specific *efnb2* knockout mice in this same study exhibited only a slight increase in osteoclast number without a change in bone volume,

osteoblast surface, or bone mineral density (Zhao *et al.* 2006). In contrast, a myeloid lineage *efnB1* knockout mouse also driven by *Lys2-cre* expression did present a phenotype with increased resorption (Cheng *et al.* 2012), which suggests that *efnB1* is a negative regulator of osteoclast function and that there is a redundancy in the *efnBs*, and possibly the Ephs, in osteoblast–osteoclast coupling. In either case, a functional demonstration of forward or reverse signaling in osteoblast–osteoclast coupling, respectively, would require that the phenotype be accompanied by a reduction in signaling from an EphB receptor in an osteoclast-specific *efnB* knockout or *efnB*-specific signaling to a specific receptor in an osteoblast-specific *Ephb* knockout.

In vitro, *efnB2* expression and *efnB2*–EphB4 interaction in response to PTH or PTHrP treatment has been associated with the differentiation of stromal cells to mineralizing osteoblasts (Allan *et al.* 2008). EphB4 inhibition by soluble EphB4 inhibited *efnB2*–EphB4 signaling and the expression of late-stage osteoblast differentiation markers and promoted osteoclast formation, even preventing the anabolic response to PTH. This effect required osteoblasts *in vitro*, indicating that signaling between *efnB2* and EphB4 within the osteoblast lineage is required for osteoblast differentiation and osteoclast formation (Takyar *et al.* 2013). These results suggest that *efnB2*–EphB4 regulation of bone formation and resorption might be complicated by the expression and function of multiple *efns* and Ephs during the development of the bone cell lineages. Indeed, this interaction extends to skeletal pathology in the adult; elevated EphB4 expression in osteoarthritis chondrocytes was reduced by an *in vitro* application of *efnB2*, which increased Col-II gene expression, a marker of chondrocyte development, and decreased markers of bone resorption (Kwan Tat *et al.* 2009).

In addition to its interactions with EphB4 in bone homeostasis, *efnB2* is important in calvarial bone formation (Benson *et al.* 2012). Embryonic expression of *efnB2* is found in the calvarial sutures and periosteum but expression is confined to the sutures of the adult murine calvariae. It promotes the expression of bone formation markers *in vitro* and calvarial bone formation in organ culture, possibly through EphB1 and EphB2 signaling. *In vivo*, *efnB2* might mediate bone formation through Ephs outside of the *efnB2*–EphB4 signaling circuit, as it promotes bone formation in the calvariae, where EphB4, its preferred receptor, is not expressed.

Other *efnB* ligand–receptor signaling combinations could regulate osteoblast functions through osteoclasts. The anti-resorptive alendronate has been demonstrated to increase the expression of *efnB1*, EphB1, and EphB3 in mice and decrease bone sialoprotein and osteonectin, markers of bone formation that were dependent on pre-osteoclasts. Because the expression of *efnB1* and these Ephs was present on osteoclasts and osteoblasts, respectively, the authors conclude that *efnB1* expressed in osteoclasts inhibits osteoblast differentiation (Shimizu *et al.* 2012). This signaling appears similar to that of *efnB2* and EphB4 in maintaining bone homeostasis (Zhao *et al.* 2006) and suggests a degree of functional redundancy among the *efns* and Ephs.

Efn–Eph functions have also been associated with bone repair. In addition to its role in calvarial bone formation during development, *efnB2* expression is elevated at sites of

calvarial bone injury, suggesting regulation of the bone formation phase of bone repair (Benson *et al.* 2012). The Col-I-mediated overexpression of transgenic *Ephb4* in osteoblasts in a murine model of femur fracture healing increased osteogenic progenitors and decreased osteoclasts, which resulted in increased bone formation and reduced bone remodeling within the fracture callus (Arthur *et al.* 2013). These results might involve efnB2–EphB4 signaling, as an increase in EphB4 would be expected to decrease osteoclastogenesis predicted by reverse signaling through efnB2 and thereby increase bony callus formation (Zhao *et al.* 2006). Alternatively, EphB4 might function by forward signaling from different efnBs. Thus, interaction between efnBs in one cell type and their receptors in different cell type can lead to enrichment of various cell types that contribute to key cellular processes involved in fracture repair.

4. Interactions between IGF and Efn–Eph Signaling

There is now substantial evidence in the literature to suggest that interactions between chondrocytes, osteoblasts, and osteoclasts are important in the regulation of bone metabolism. In terms of molecular signals that contribute to the interactions between the various bone cell types, IGF-I and efn–Eph signaling have received considerable attention since studies using various knockout mouse models and *in vitro* cell culture systems demonstrate that these signaling pathways are critical in bone and are required for stimulation of bone formation by a number of key anabolic agents including PTH and mechanical strain. Therefore, it is likely that IGF-I and efn–Eph signaling pathways interact to regulate bone metabolism during normal and disease states. Accordingly, recent studies provide evidence that some of the actions of IGF-I in the skeleton are mediated via regulation of efn–Eph signaling as described below.

Recent studies support a role for IGF-I interactions with efn–Eph signaling in postnatal skeletal growth. Global *Epha4* knockout mice displayed a dramatic general reduction in body size that was gene dose-dependent in heterozygotes. Bone growth was affected; there was a reduction in the length of the epiphyseal growth plates of the long bones consistent with growth reduction and not achondroplasia. This phenotype was accompanied by low plasma IGF-I levels and markedly reduced *Igf1* mRNA in the liver and other tissues. Since GH and GHR levels were unchanged, the reduction in IGF-I production was not due to decreased GH signal. Rather, EphA4 forms a complex with the GHR and downstream GH effectors JAK2 and STAT5B to enhance IGF-I production in response to GH. This effect was largely JAK2-dependent, although some direct effect on STAT5B was observed. Thus, in the absence of EphA4, decreased production of IGF-I resulted in a small body size (Jing *et al.* 2012).

Further molecular studies characterized the binding of JAK2 and EphA4 to the GHR, the phosphorylation status of EphA4, GHR, JAK2, and STAT5B resulting from their mutual interactions, and the subsequent nuclear translocation of STAT5B. While GH canonically activates STAT5B via the GHR and JAK2, efn–EphA4 signaling was found to induce STAT5B activation independent of JAK2. The GHR was required for this JAK2-independent activation of STAT5B, although its precise role remains to be elucidated (Sawada *et al.*

2017). These studies reveal a global role for EphA4 functions in the body, in contrast to tissue-specific knockout models that demonstrate its regulation of osteoclast functions.

IGF-I is an important regulator of endochondral bone formation, and its functions have been demonstrated to be mediated by efnB2–EphB4 signaling (Wang *et al.* 2014). *In vivo*, the normal expression was reduced in *Igf1* knockout mice and in the growth plates of *Col2*- or *Osx*-specific *Igf1r* knockout mice (Wang *et al.* 2015). *In vitro*, inhibition of efnB2 by deletion or EphB4 expression with a specific receptor inhibitor reduced the expression of osteogenic markers in response to IGF-I. *In vitro* blocking of EphB2 and EphB4 interaction through specific inhibition of efnB2 or EphB4 also reduced the development of osteoclasts and osteoblasts, respectively, in response to IGF-I (Fig. 3). With respect to chondrocytes, *in vitro* inhibition of the efnB2–EphB4 interaction reduced the expression of markers of chondrocyte and osteoclast development in response to IGF-I. These results provide strong evidence for efnB2–EphB4 functions in cell–cell contact for IGF-I-induced bone development and for intermittent PTH-induced bone development, as PTH regulates IGF-I functions.

There is also now evidence in the literature that IGF-I can interact with efn–Eph signaling to regulate cellular processes. For example, IGF-I has been shown to regulate efnB1 functions following tooth injury. Tooth injury in mice with dentin matrix protein (*dmp*)-1-driven deletion of *Igf1r* demonstrate reduced *efnb1* expression and impaired tooth repair. In response to the dental pulp capping procedure, *Igf1* expression was increased. However, specific inhibition of IGF-IR signaling pathways revealed that *efnb1* and *Ephb2* expression was mediated by different pathways. EfnB1 and EphB2 signaling therefore mediate IGF-I functions during tooth repair (Matsumura *et al.* 2017).

Skeletal myogenesis requires the activation of the PI3K/Akt cascade but is inhibited by the ERK1/2 cascade. IGF-I induces myogenesis; however, IGF-I is known to activate both the PI3K/Akt and ERK1/2 cascades. EfnA–EphA signaling downregulates the Ras/ERK1/2 pathway. *In vitro* activation of efnA1 enhanced the ability of IGF-I to increase myogenic differentiation, an effect which was dependent upon the ability of EphA to decrease ERK1/2 signaling. Furthermore, the ability of IGF-I to induce myogenesis was inhibited by downregulation of efnA–EphA, an effect which was rescued by inactivation of ERK1/2. EfnA–EphA-mediated suppression of ERK1/2 is thus required for IGF-I to induce myogenesis (Minami *et al.* 2011).

Studies of protein associations also have benefited from the development of databases that predict interactions between proteins. Significant changes in gene expression obtained from microarray data can be merged with protein–protein interaction (PPI) databases to identify proteins that might interact to regulate the condition. By merging miRNA microarray data and PPI predictions, IGF-I and EphA4 proteins also were identified within two separate subnetworks of interacting proteins in OA (Wang *et al.* 2013). Although a functional connection between IGF-I and EphA4 was not investigated, their presence in the OA PPI network suggests an interaction between them is possible and that such a database approach might be valuable in identifying candidate proteins that could interact with large and widely expressed families of genes, such as the efn and the Ephs.

Recent investigations have only begun to characterize the interactions of IGF-I, efn, and Ephs in the skeleton. Given the importance of IGF-I as a growth factor in several tissues and the widespread expression and importance of the efn and Ephs in tissue development, it is probable the efn and Eph interactions with IGF-I, as well as other growth factors, are extensive and remain to be elucidated.

5. Summary

The IGF-I and efn–Eph signaling pathways are both critical regulators of bone cell function and skeletal development and maintenance. Many different signals and factors regulate IGF-I signaling at the systemic and local levels, making it a point of integration for several different pathways which induce bone growth and maintenance. Efn–Eph signaling is a complex system which, particularly through cell–cell interactions, contributes to the development and differentiation of many bone cell types. Thus, the fact that recent studies have established the interaction between these two signaling pathways opens a vast array of new opportunities for investigation into the mechanisms of and potential therapies for skeletal conditions such as osteoporosis and fracture repair.

6. Future Research Directions

While the role of IGF-I signaling in bone has been studied in great detail, the role of IGF-II in human bone metabolism remains to be elucidated. In rodents, IGF-II is primarily a fetal growth factor, and its expression declines during postnatal growth while IGF-I expression increases. In contrast, IGF-II is expressed at higher levels in adult human tissues including bone. The question of whether IGF-II participates significantly in adult bone metabolism and osteoporosis pathogenesis thus remains to be established. Furthermore, while recent evidence suggests a role for IGF-I in age-related bone loss and osteoporosis, the extent of its contribution and usefulness as an anabolic therapy for osteoporosis deserve further consideration and study. Additionally, work remains to be done in elucidating the particular roles of the various efn and Ephs in each of the bone cell types and their contributions to the regulation of skeletal growth. The precise roles of efn–Eph and IGF-I signaling in fracture repair also remain to be uncovered. Finally, the mechanisms by which IGF-I and efn–Eph signaling interact to regulate skeletal metabolism and growth must be determined with an emphasis towards developing novel strategies for treatment of osteoporosis and bone repair.

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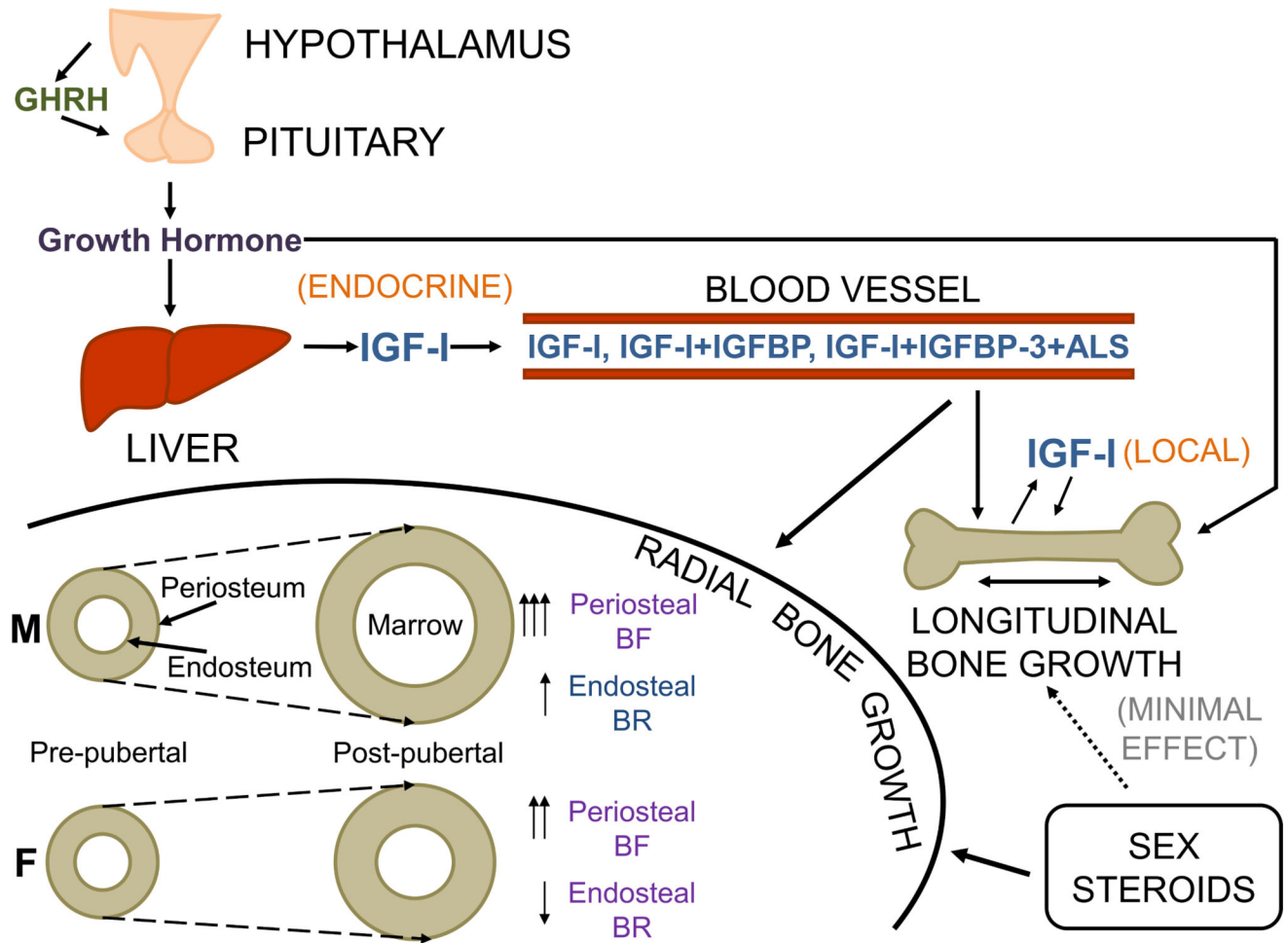


Figure 1. A model of GH/IGF-I and sex hormone signaling interacting to regulate bone growth GH effects on the skeletal system is mediated via both endocrine and local mechanisms through hepatic IGF-I and local modulation of IGF-I expression in bone cells, respectively. Sex steroids contribute relatively little to longitudinal bone growth, but they interact with IGF-I signaling to regulate radial bone growth. The interaction of IGF-I and sex steroid signaling results in skeletal sexual dimorphism in which, during pubertal growth, male (M) periosteal bone formation (BF) and endosteal bone resorption (BR) are both increased, producing bones with a larger diameter as well as a larger marrow cavity, while female (F) periosteal BF is increased but endosteal BR is decreased, leading to smaller bones with more preservation of cortical bone (Adapted with permission from Lindsey and Mohan, 2015).

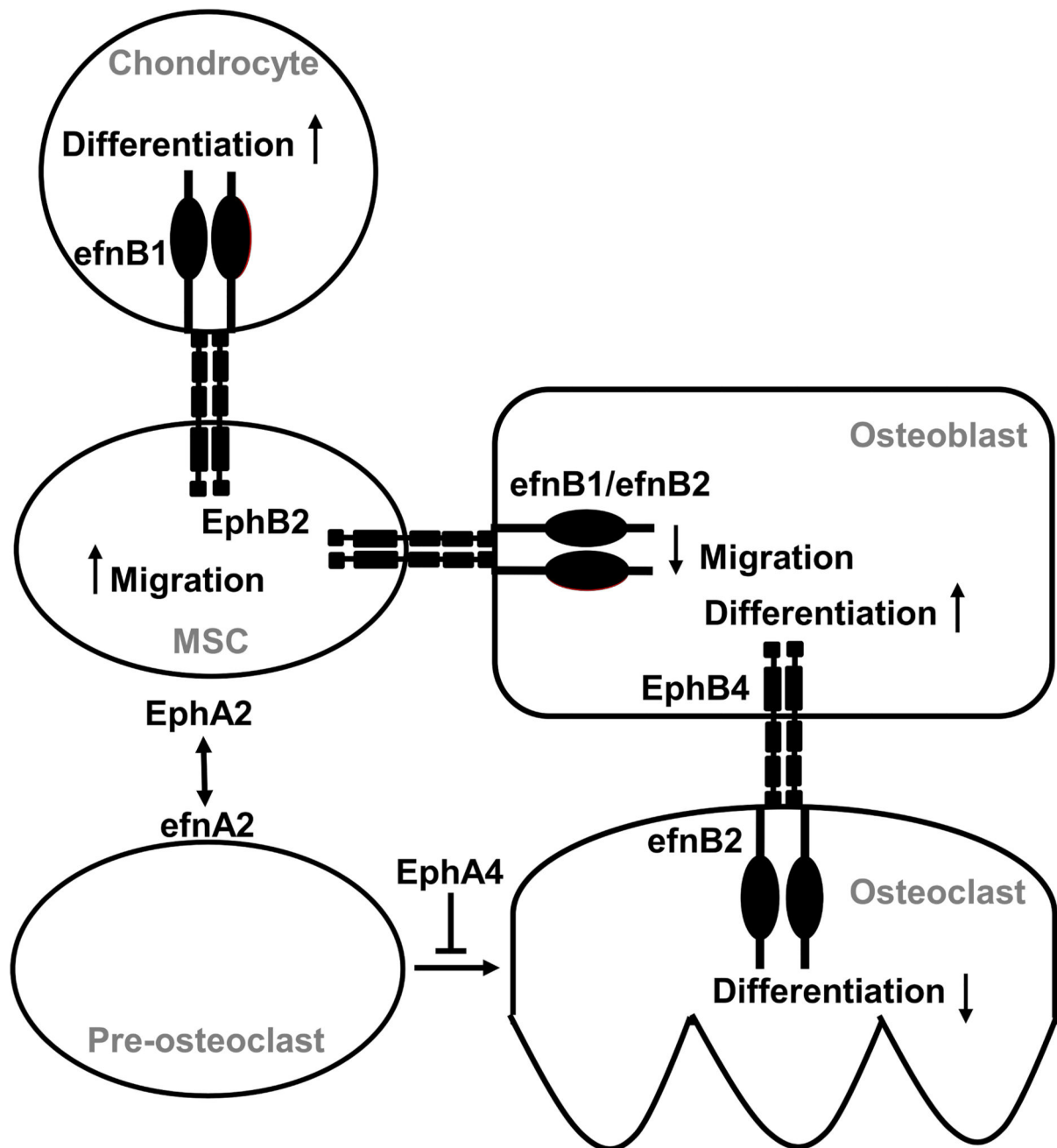


Figure 2. A model for efn–Eph regulation of bone cell interaction in bone development and homeostasis

Several efn and Ephs mediate growth plate development during bone growth. Interactions between osteoblasts (OB), osteoclasts (OC), mesenchymal stem cells (MSC), and chondrocytes are mediated by different ligand–receptor combinations, coordinating bone cell development and activity through forward and reverse signaling. Osteoblast development is mediated by forward signaling through EphB4 from efnB2 (Zhao *et al.* 2006) and reverse signaling through efnB1/efnB2 from EphB2 (Arthur *et al.* 2011). Osteoclast development is mediated by efnA2 and EphA4 (Irie *et al.* 2009; Liu *et al.* 2017) as well as through reverse signaling from EphB4 through efnB2 (Zhao *et al.* 2006).

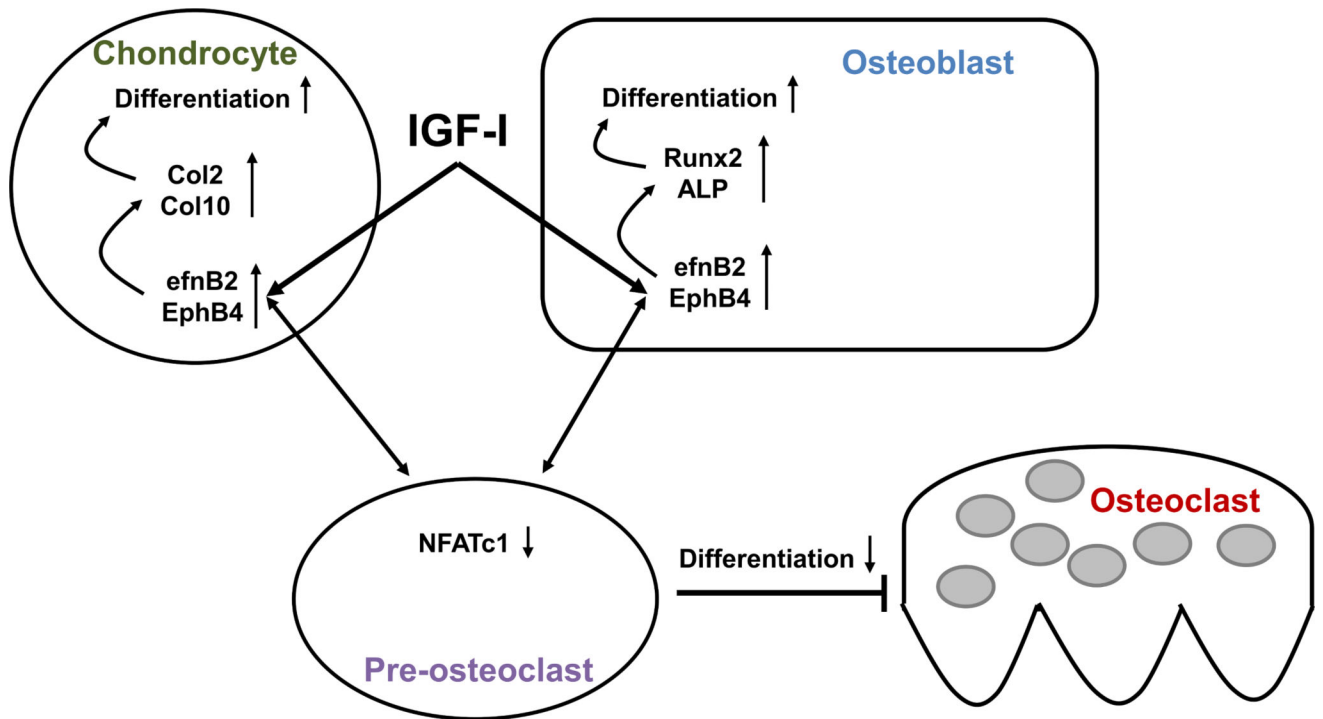


Figure 3. A model of the interaction of IGF-I and efn–Eph signaling in skeletal metabolism IGF-I can act directly on chondrocytes and osteoblasts, increasing efnB2–EphB4 signaling and leading to differentiation of each cell type by increasing expression of key transcription factors. Through reverse signaling, efnB2–EphB4 interaction can decrease NFATc1 expression in osteoclast precursors and inhibit formation of differentiated, mature multinucleated osteoclasts (Zhao *et al.* 2006; Kwan Tat *et al.* 2009; Wang *et al.* 2015).